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The novel UGT1A9 intronic I399 polymorphism appears as a predictor of SN-38 glucuronidation levels in the liver.

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Running title: UGT1A9 polymorphisms and SN-38 hepatic glucuronidation

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ABSTRACT

Polymorphisms in *UGT1A9* were associated with reduced toxicity and increased response to irinotecan in cancer patients. To clarify the role of *UGT1A9* variants on the *in vitro* glucuronidation of SN-38, UGT protein expression, glucuronidation activities for SN-38 and probe substrates of the UGT1A9 and UGT1A1 were measured in 48 human livers. Genotypes were assessed for *UGT1A9* (-2152C>T, -275T>A, -118$T_{9>10}$), three novel *UGT1A9* variants *(*-5366G>T, -4549T>C and I399C>T) and *UGT1A1* (-53$TA_{6>7}$, -3156G>A and -3279T>G). Of all variants, the *UGT1A9* I399C>T was associated with the most dramatic change in SN-38G (2.64-fold; p=0.0007). Compared to *UGT1A9 I399C/C,* homozygous *I399*T/T presented elevated UGT1A1 and UGT1A9 proteins and higher glucuronidation of UGT1A9 and UGT1A1 substrates ($p<0.05$). The very low LD ($r^2<0.19$) between *UGT1A9 I399* and all other *UGT1A1* and *UGT1A9* variants suggests a direct effect or linkage to unknown functional variant(s) relevant to SN-38 glucuronidation. The *UGT1A9*-118$T_{9/10}$ was also linked to alteration of SN-38 glucuronidation profiles in the liver ($p<0.05$) and associated with higher UGT1A1 protein (p=0.03). However, *UGT1A9-118*T_{10} appears to have low functional impact due to the lack of correlation with UGT1A9 protein levels and a modest 1.4-fold higher reporter gene expression associated with the -*118*T_{10} allele in HepG2 cells (p=0.004). In contrast, the *UGT1A9* -5366T, -4549C, -2152T and -275A, associated with higher UGT1A9 protein (2-fold; $p<0.05$), have no influence on SN-38G. Despite limitations due to sample size, results indicate that *UGT1A9* I399 and -*118*$T_{9/10}$ may represent additional candidates in combination with *UGT1A1* promoter haplotypes for the prediction of SN-38 glucuronidation profile *in vivo.*

Irinotecan (CPT-11) is a topoisomerase inhibitor used to treat metastatic colorectal cancer (Douillard et al., 2000; Saltz et al., 2000). Its metabolism is complex and includes oxidation by cytochrome P450 and activation by carboxylesterase to form the active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) (Kawato et al., 1991). Glucuronidation catalyzed by UDP-glucuronosyltransferases (UGT) represents the major inactivation pathway for SN-38 (Gupta et al., 1994). Of the 16 functional human UGT enzymes, UGT1A1 and UGT1A9 were identified as the main hepatic enzymes involved in the inactivation of SN-38 to SN-38-glucuronide (SN-38-G) (Hanioka et al., 2001; Gagne et al., 2002). UGT1A1 and UGT1A9 are encoded by a single gene by exon sharing of individual exons 1 with common exons 2–5, while at least 6 other UGT1A proteins originate from this gene (Gong et al., 2001).

Several studies revealed the importance of UGT1A1 and UGT1A9 in the hepatic metabolism of SN-38 (Iyer et al., 1998; Gagne et al., 2002; Carlini et al., 2005). A number of polymorphisms in *UGT1A1* and *UGT1A9* affecting expression and protein function have been identified and could potentially modulate the metabolism of SN-38 *in vivo* (Zheng et al., 2001; Guillemette, 2003; Villeneuve et al., 2003; Girard et al., 2004; Yamanaka et al., 2004) as well as response to irinotecan-based chemotherapy (Ando et al., 2000; Innocenti et al., 2004; Marcuello et al., 2004; Rouits et al., 2004; Carlini et al., 2005).

Among these genetic variations, patients with the *UGT1A1*28* variant, associated with Gilbert's syndrome (Bosma et al., 1995), show higher levels of SN-38 and experienced

severe diarrhea and neutropenia (Ando et al., 2000; Innocenti et al., 2004; Marcuello et al., 2004; Rouits et al., 2004). Additional polymorphisms in the *UGT1A1* gene have been reported and linked to a variable SN-38 glucuronidation, namely the *-3156 G>A* and the *-3279 T>G* variations (Innocenti et al., 2002; Sugatani et al., 2002). Genotyping of these variations in the promoter region of *UGT1A1* along with the -53 variant, has been suggested to improve the prediction of UGT1A1 status and specific *UGT1A1* haplotypes are associated with altered SN-38 phenotypes (Innocenti et al., 2002; Sai et al., 2004; Innocenti et al., 2005) (Kitagawa et al., 2005).

Since the UGT1A9 protein is involved in the formation of SN-38G (Hanioka et al., 2001; Gagne et al., 2002), we recently identified polymorphic variations in the Caucasian population associated with altered gene expression or catalytic efficiency, namely the *-2152C>T, -275T>A* and $M^{33}T$ (*98T>C*) (Villeneuve et al., 2003; Girard et al., 2004). Another group reported that the *-118*$T_{9>10}$ repeat was associated with higher reporter gene expression in HepG2 liver cells (Yamanaka et al., 2004) and this variant was recently linked to higher incidence toxicity and increased response to irinotecan in cancer patients. (Carlini et al., 2005). Still, definitive conclusions on the exact role of *UGT1A1* and *UGT1A9* variations and their influence on irinotecan response in patients cannot be drawn. Additional studies are critical for determining the role of other polymorphisms in UGTs in addition to *UGT1A1* and especially, the importance of *UGT1* haplotypes.

In this study, we wanted to gain insights into the role of polymorphisms in the *UGT1A9* in modulating SN-38 glucuronide formation in the liver. *UGT1A9* was resequenced up to

-5.5 kb of the ATG and included intronic sequences. UGT1A1 and UGT1A9 protein content was measured to identify polymorphisms and haplotypes/diplotypes that might influence gene expression and SN-38G formation in liver microsomes.

MATERIALS AND METHODS

Reagents

SN-38 was prepared by hydrolysis of irinotecan-HCL (McKesson, ON, CA). Identity of the SN-38 was assessed by mass spectroscopy and purity was assessed by U.V. absorbance spectra. Bilirubin, estradiol, and estradiol-3-glucuronide were purchased from Sigma-Aldrich (St Louis, MO).

Genomic DNA and liver samples

Human genomic DNA and liver samples were obtained from different sources as described previously (Court et al., 2002) as approved by the respective human research institutional review boards (CHUQ research center, Laval University and Tufts University). Available subject information included gender, age, race, ethnicity, and histories of smoking and alcohol use, as described in Hesse et al. (Hesse et al., 2004). The quality of the liver samples was ascertained by reference to at least 10 other glucuronidation activities measured using the same set of livers. Livers that consistently showed low activity values (>2-fold lower for all measured activities) relative to the median activity value for the entire liver set had been excluded from study.

Genotyping of *UGT1A1* and *UGT1A9* polymorphisms

UGT1A1 -53 $TA_{6>7}$, -3156 G>A and *-3279 T>G* genotypes for all 48 sujects were determined in a previous study by Girard et al. (Girard et al., 2005). Genotyping of *UGT1A9* exon 1 polymorphisms at codon 3 and 33 and 11 promoter polymorphisms from -87 to -2152, were performed previously (Girard et al., 2004). For the discovery of new variants, primers R503, F506, R507, F838, R839, F840, R841 and F842 (Table 1) were used to amplify the *UGT1A9* promoter region located between nucleotides -1654 and -5525 (relative to the ATG). Genotyping of the two new polymorphisms located at positions -4549 and -5366 was performed by direct sequencing. Primers F1159/R1164 and F1154/R1165 were used to amplified regions with polymorphisms -4549 and -5366 respectively. The PCR products were submitted to sequencing using primer R1164 and F1154. The UGT1A9 intron polymorphisms at positions *143C>T*, *152G>A, 201A>C*, *219T>A*, *313A>C* and the novel *399C>T* (relative to the end of *UGT1A9* exon 1) were genotyped by direct sequencing, with primer F1293, of a PCR product generated with primers F1293 and R1298.

Construction of plasmid and transfections

Primers F1121 and R670 were used to amplify the proximal promoter region of *UGT1A9* between -152 and +2 (relative to the ATG). The PCR product was digested with HindIII and Xho1 and inserted in the pGL3 vector. Primers F1473 and R1474 were also used to amplify the proximal promoter region of *UGT1A9* between -171 and -1 (relative to the ATG). The PCR product was digested with SmaI and inserted in the pGL3 vector. A variant sequence was generated by site directed mutagenesis for both strategies

(QuikChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) with primers F1155 and R1166.

HepG2 cells were obtained from American type and culture collection (Manassas, VA) and were grown in minimum essential medium (MEM). Medium was supplemented with 20% fetal bovine serum (Wisent, St-Bruno, Quebec, Canada), as well as a 0.1-mmol/Lmixture of nonessential amino acids, 100 IU/mL of penicillin, and 50 µg/mL of streptomycin (all from Invitrogen, Burlington, Ontario, Canada). Sodium pyruvate at a final concentration of 1mM was added. Transfections were performed as described previously (Duguay et al., 2004a). Cells were harvested 24h post transfection and assayed for promoter activity using the Dual luciferase reporter assay system according to the manufacturer's recommendations and that includes as an internal control, the pRLnull vector (encoding Renilla luciferase) cotransfected in each wells (Promega, Madison, WI). Luciferase activity was measured using 40 µl of cell lysates in a 96-well plate on a microplate luminometer LB96V (EG&G Berthold, Germany) and normalized to the Renilla luciferase values. Means of duplicate luciferase activity of four independent experiments were compared between the different constructs with a two-tailed unpaired Student's t-test.

UGT1A1 and UGT1A9 protein quantification and enzymatic assays

UGT1A1 and UGT1A9 protein quantification in all 48 liver microsomes was performed by semi-quantitative western blot using specific antibodies for UGT1A1 and UGT1A9 as described previously (Duguay et al., 2004b; Girard et al., 2004). The relative levels of UGT proteins content were compared to the sample with the lowest expression. SN-38 enzymatic assays were performed in a final volume of 100 µL containing 50 mM Tris-

HCl PH 7.5, 10 mM $MgCl_2$, 2 mM UDPGA, 5 μg/ml pepstatine, 0.5 μg/ml leupeptine, 10 μg/ml phosphatidylcholine, alamethicin (0.2 mg/ml) (Sigma-Aldrich Chemical Co., Oakville, ON, Canada), 0.5 mM EDTA, 5 μM SN-38 and 8 μg of liver microsomes from each subject, as previously described (Gagne et al., 2002). Reactions were stopped by the addition of 100 μL methanol 2% HCL 2N. SN-38G detection and quantification were performed by LC/MS/MS as described previously (Gagne et al., 2002; Villeneuve et al., 2003). Estradiol 3-glucuronidation activities were determined as previously described (Krishnaswamy et al., 2003). For bilirubin glucuronidation activities, 100 uL incubations were prepared containing 50 mM phosphate buffer (pH 7.5), 10 ug liver microsomal protein, 10 uM bilirubin (with 2% DMSO as a solubility enhancer), 0.5 ug alamethicin, and 5 mM UDPGA. Because of light sensitivity, bilirubin stock solutions (0.5 mM in DMSO) were prepared fresh each day and protected from light, while incubations were performed in 0.5 mL amber tubes under minimal light conditions. Incubations were performed for 10 min in a 37°C water bath, terminated by addition of 50 uL cold acetonitrile containing 5% glacial acetic acid and 5 nmoles of 3'-azidothymidine (as internal standard), and then vortexed. After centrifuging for 10 min at 14,000g, the supernatant was transferred to HPLC tubes, dried down in a vacuum oven set at 45°C, and reconstituted with 100 uL of 0.1% trifluoroacetic acid (TFA) in water prior to HPLC analysis. HPLC (Agilent 1100 system) was performed using a 4.6×250 mm 10um C18 column (Synergi Hydro-RP, Phenomenex, Torrance, CA) and effluent monitored using a UV absorbance diode array detector set at 450 nm (bilirubin and glucuronide) and 254 nm (internal standard). Mobile phase run at 1 mL/min consisted of 0.1% TFA with 10% acetonitrile in water initially, with a linear gradient to 100% acetonitrile over 20 min, and

held at 100% acetonitrile for a further 10 min before returning to initial conditions over 5 min. Internal standard, bilirubin glucuronide, and bilirubin eluted at 8, 15 and 28 min, respectively. Glucuronide peak identity was confirmed by HPLC-MS (ThermoFinnigan DecaXP-Plus, Thermo Electron, Somerset, NJ) and sensitivity to treatment with β-glucuronidase and alkaline pH adjustment. Since bilirubin glucuronide was not available for use as a standard, quantitation was achieved by use of a standard curve generated using known bilirubin concentrations assuming similar UV absorbance of substrate and glucuronide. Results were expressed as nmole equivalents of bilirubin glucuronide formed per minute per mg microsomal protein. Preliminary studies confirmed linear metabolite formation for up to 30 min and 0.35 mg /mL microsomal protein concentration. Both within and between assay variations was less than 15%.

Haplotype and linkage analyses

Haplotypes for *UGT1A1* and *UGT1A9* were determined using Phase v2.1 program (Stephens et al., 2001) and analysis were performed with Caucasian subjects only (n=42). The linkage between the different polymorphisms was determined with the LDplotter tool program found at https://innateimmunity.net/. All statistical analyses were done using the JMP V4.0.2 program (SAS institute, Cary, NC) and the SigmaStat program (version 3.1; SPSS Inc., Chicago, IL). Correlation analyses between activities values were done using the Spearman's test. Rs value higher than 0.50 and p value less than 0.05 were considered significant. One-way ANOVA and a comparison for each pair using Student't tests were used to determine the relationship between genotypes/diplotypes and protein levels or glucuronosyltransferase activities. Only, diplotypes with more than 3 subjects were included in the analysis. The normal distribution of all expression and

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activities values was evaluated with the Shapiro-Wilk W test ($p>0.05$). Raw data that were not normally distributed were transformed with a logarithm function to achieve a normal distribution that is essential for parametric test. For all analyses, a p value less than 0.05 was considered significant.

RESULTS

To generate a comprehensive basis for investigating the role of *UGT1A9* polymorphisms on SN-38 glucuronidation, we firstly extended the search for genetic polymorphisms to 5.5 kb of upstream sequence and included intronic sequences. We report two novel promoter SNPs, *-5366G>T* and *-4549T>C* (Fig.1), in strong linkage disequilibrium with the previously reported functional variations *–2152C>T* (LD; $r^2=1.0$) and *–275T>A* (LD; $r^2=0.65$). All four polymorphisms are associated with significantly higher hepatic UGT1A9 protein expression in heterozygous subjects (1.6-fold; Table 2). The previously reported functional *UGT1A9* -118$T_{9/10}$ variant (Yamanaka et al., 2004), was also observed at a frequency of 0.4. Intronic variants recently identified at positions *I152A*, *I219A* and *I313C* (Carlini et al., 2005), were found in the population tested at an allelic frequency between 0.13 to 0.4) whereas the *I143T* and *I201C* were not observed. A novel *UGT1A9* intronic variant at position *I399C>T* was found at a frequency of 0.44.

Correlation analyses were then performed with enzymatic activities, UGT protein content in livers and *UGT1A9* polymorphisms. Compared to individuals with the *UGT1A9 I399C/C* genotype, subjects with the *UGT1A9 I399T/T* genotype had a significant increase in SN-38G formation ($p<0.05$; Fig. 2a). In addition, UGT1A1 and UGT1A9

hepatic protein content were elevated in this group of subjects ($p<0.05$; Fig. 2b,c). This elevation in UGT protein content was reflected at the level of glucuronidation activities. Glucuronidation of UGT1A1 substrates, namely bilirubin and estradiol-3, as well as UGT1A9 substrates, MPA and propofol (Fig.2d), were significantly increased in subjects with the *UGT1A9 I399T* allele ($p<0.05$; Table 2).

In the population tested, the intronic *UGT1A9 I399T* variant was not linked to the *UGT1A1 -53*, *-3156* and *-3279* variants ($r^2<0.06$; Fig. 3). In addition, a very low degree of LD exist between *UGT1A9 I399T* and all other UGT1A9 functional variants ($r^2<0.18$) in the subjects tested (Fig. 4). When restricted to subjects with the *UGT1A1 -53(TA)$_{6/6}$* genotype, individuals with the *UGT1A9 I399T/T* genotype presented 1.7-fold higher UGT1A1 protein content and 2.5-fold higher SN-38 glucuronidation activity compared to *UGT1A9 I399C/C* carriers ($p<0.05$, Fig. 4). Due to the limited sample size, we could not verify the effect of the *UGT1A9* I399 genotype within homozygous subjects for the *UGT1A1-53(TA)$_{7/7}$* genotype. Similar results were observed for bilirubin and estradiol glucuronide levels (1.8-fold and 2.0-fold; $p<0.05$; data not shown). These observations suggest that the *UGT1A9 I399T* variant may be functional or linked to an unknown functional variant in the *UGT1* gene affecting SN-38 glucuronidation levels.

The *UGT1A9 -118T$_{10}$* variant was also significantly associated with higher rates of SN-38G formation ($p=0.05$) (Table 2; Fig.5a). Despite the fact that this polymorphism was not significantly associated with variation of hepatic UGT1A9 protein content ($p>0.05$), homozygous *-118T$_{10}$/T$_{10}$* individuals showed significantly higher levels of UGT1A1 protein compared with individuals *-118T$_9$/T$_9$* ($p<0.05$; Fig.5c). Consistent with this

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observation, the -118T_{10} variant was associated with higher glucuronidation of UGT1A1 substrates, estradiol and bilirubin ($p<0.05$; Table 2). On the other hand, the *UGT1A9* -*118T_{10}* was not associated with significant alteration of the glucuronidation of UGT1A9 specific substrates (propofol and MPA) (Girard et al. 2004), suggesting an indirect effect of this variation on UGT1A1 expression and UGT1A1-mediated glucuronidation activity. To further evaluate this hypothesis, HepG2 cells derived from human liver cells were transfected with various constructs of the *UGT1A9* promoter containing 9 or 10 T repeats at position -118. Results revealed a modest functional impact of 1.4-fold higher expression associated with the -*118T_{10}* compared to the -*118T_9* ($p=0.004$; ; Fig.5d). This modest increase may explain the lack of significant alteration of the UGT1A9 protein content and UGT1A9-mediated glucuronosyltranferase activities in liver microsomes.

In the population studied and when restricted to Caucasian subjects (n=42), a low degree of LD was observed between the *UGT1A9 I399 C>T* and the -*118 $T_{9/10}$* variant ($r^2=0.19$). In contrast, the *UGT1A9 -118T_{10}* was found to be linked to the *-3279G* variant of *UGT1A1* ($r^2=0.40$; Fig. 3). This linkage may explain part of the effect of the *UGT1A9* -*118T_{10}* allele on UGT1A1 protein and activity levels. Of interest, LD was relatively low between *UGT1A1 -53* and *UGT1A9 -118T* ($r^2=0.26$).

Fig. 6 shows the results of *UGT1A9* diplotype analyses, restricted to polymorphisms significantly associated with altered protein expression or glucuronosyltranferase activities (-5366, -4549, -2152, -275, -118 and I399). The haplotype UGT1A9_I (GTCT9C) is the most frequent (0.46) and correspond to the reference *UGT1* sequence

(AF297093; Fig. 1). Haplotype UGT1A9_II (GTCT10T), with the variant *-118T$_{10}$* and *I399T*, is the second most frequent haplotype (0.33). Haplotype UGT1A9_III (GTCT9T), characterized I399T, is also common in the Caucasian population (0.13). Three other haplotypes (UGT1A9_IV, V and VI) were found but at a relatively low frequencies (0.05, 0.03 and 0.01). There was no significant association between *UGT1A9* diplotypes and UGT1A9 hepatic protein content (Fig.6a). However, compared to subjects with the UGT1A9_I × UGT1A9_I reference diplotype, subjects with the UGT1A9_II × UGT1A9_III diplotype presented higher SN-38 glucuronidation activities ($p<0.05$; Fig.6b). The impact of the haplotypes characterised by the presence of the *-5366T, -4549C, -2152T* and *-275A* polymorphisms could not be assessed because of their low frequencies.

We then evaluated the impact of the *UGT1A1* diplotypes (Fig.7). Firstly, we observed that the *UGT1A1* variants *-53 (UGT1A1*28, TA$_7$), -3156G>A* and *-3279T>G* were individually associated with lower UGT1A1 protein content and decreased glucuronidation activities for SN-38 as well as for UGT1A1 probe substrates bilirubin and estradiol (Table 2). Compared to diplotype UGT1A1_I (TG6) x UGT1A1_I, a significant association of diplotype UGT1A1_II (GA7) x UGT1A1_II with UGT1A1 protein content and SN-38 glucuronidation was shown consistent with previous reports ($p<0.05$; Fig.7b, c) (Innocenti et al., 2004). This relationship was also significant for bilirubin and estradiol (data not shown). A gene-dosage effect could be appreciated for UGT1A1 protein content and SN-38G with an intermediate level of protein or glucuronide for livers with diplotype UGT1A1_I x UGT1A1_II.

Finally, although our samples size was limited, these results led us to explore the haplotype structure of *UGT1A1* and *UGT1A9* variants in the Caucasian subjects tested. Ten unambiguous *UGT1A1-1A9* haplotypes were inferred. Livers with diplotype UGT1A1-1A9_II (GTCT9CGA7) x UGT1A1-1A9_II had a lower, but not significant, UGT1A1 protein level and significantly lower glucuronidation activities for SN-38 ($p<0.05$) (Fig 8), bilirubin and estradiol ($P<0.05$) (data not shown) with intermediate levels for livers with diplotype UGT1A1-1A9_I x UGT1A1-1A9_II. No significant association of diplotypes with UGT1A9 protein content was detected.

DISCUSSION

Findings demonstrate that the *UGT1A9 I399* and *-118* polymorphisms have an influence the hepatic glucuronidation of SN-38 *in vitro* and that their effects are likely indirect or through their linkage to unknown functional *UGT1* variant(s).

The presence of a previously unreported variation in the intron 1 of *UGT1A9* (*I399C>T*) is highly predictive of SN-38 glucuronidation rates by human liver microsomes. In fact, this variant was associated with the most significant and most dramatic increased in SN-38G activity (2.64-fold; $p=0.0007$) compared to all other genotypes including those of the *UGT1A1* promoter. The presence of the *UGT1A9 I399T* allele is also highly predictive of UGT1A1 protein content in the liver, rates of glucuronidation of specific probe substrates of the UGT1A1 protein in addition to UGT1A9 protein expression and UGT1A9-mediated activities. Furthermore, that would indicate that this variation might be an

independent predictor of SN-38 glucuronidation since within subjects with *UGT1A1* -53$(TA)_{6/6}$ genotype, those with the *UGT1A9* I399 T/T presented 2.5-fold higher SN-38 glucuronidation activity (p=0.016), compared to those with the I399 C/C. However, because of the limited liver samples in our bank, we were unable to assess this relationship in subjects homozygous *UGT1A1 -53(TA)$_{7/7}$*. Besides, these phenotypic effects do not appear to be explained by linkage to other functional variants since LD was very low between the *UGT1A9 I399* and the *UGT1A1 –53 (TA)$_n$, -3156* and *-3279* promoter variants (r^2 <0.06) and with other *UGT1A9* variants. The direct functional effects of the intronic variant could not be assessed in this study but findings suggest that it might be functionally important or link to a functional variant(s) likely unknown because of the lack of LD (r^2<0.19) with known common functional *UGT1A1* and *UGT1A9* polymorphisms.

Two novel *UGT1A9* polymorphisms in the 5'-regulatory region identified at positions -5366 and -4549 were found and are tightly linked to those previously reported at positions -2152 and -275. In previous *in vitro* experiments, *-2152T* and *-275A* variants were shown to correlate with higher UGT1A9 hepatic protein content and an increased *in vitro* glucuronidation activities for specific UGT1A9 substrates (Girard et al .2004). In addition, a recent study in renal transplant recipients established the *in vivo* functional significance of these variants by measuring a reduced MPA exposure in patients carrying these polymorphisms (Kuypers et al., 2005). Despite *in vitro* and *in vivo* evidences of their functionality, these genetic variations of the *UGT1A9* gene appear to have no significant effect on the hepatic glucuronidation of SN-38. Overall, findings suggest that

the modulation of the UGT1A9 protein in the liver would have a limited impact on the glucuronidation of SN-38.

A recent study indicated that the *UGT1A9 –118T$_{9/9}$* genotype is associated with a lower incidence of toxicity and a better response in colorectal cancer patients treated with an irinotecan-based regimen (Carlini et al., 2005). In our study, the *UGT1A9 -118T$_{10}$* allele was associated with elevated SN-38G formation in livers, higher UGT1A1 protein content and elevated bilirubin and estradiol glucuronidation. However, it still remains unclear if this polymorphism is functional. Firstly, it was found that the *UGT1A9 –118T$_{10}$* allele is tightly linked to the high activity *UGT1A7*1* and *UGT1A1*1* alleles (Carlini et al., 2005). In the liver samples from Caucasians, we confirm that the *UGT1A9 –118T$_{10}$* is in strong LD with the *UGT1A1*1* allele and associated with the high activity haplotype I of *UGT1A1* (Haplotype I TG6). This linkage might explain the relationship between the *UGT1A9 -118 v*ariant and irinotecan response rather than reflecting a direct effect of the *-118T* polymorphism on UGT1A9-mediated glucuronidation of SN-38. Consistent with this hypothesis, livers homozygous for the *–118T$_{10}$* allele demonstrated no significant changes in UGT1A9 protein content and UGT1A9-mediated glucuronidation activities compared to homozygous for the *–118T$_{9}$* allele. In contrast, subjects with the *–118T$_{10}$* presented significantly higher levels of UGT1A1 protein and glucuronidation activities of UGT1A1 specific substrates, sustaining the hypothesis of an indirect effect.

In addition, results of *in vitro* gene reporter assays in liver cells support a limited impact of the *UGT1A9 –118T$_{10}$* variation on transcriptional gene activity (1.4-fold increased; p=0.004) that may not be sufficient to influence levels of UGT1A9 protein in the liver.

This is in contrast with a previous study reporting a 2.6-fold greater transcriptional activity associated with a *UGT1A9 -118T*$_{10}$ promoter construct compared to *-118T*$_{9}$ in HepG2 liver cells. These inconsistencies between studies are somehow difficult to explain since the transfected cell line and the *UGT1A9* promoter constructs were identical in both studies. Therefore, with the aim to gain further information from this *in vitro* approach, we subcloned a slightly different promoter region including the *UGT1A9 -118* variation and obtained identical results with a 1.4-fold elevation of the reporter gene expression associated the *-118T*$_{10}$ allele. These *in vitro* observations are in agreement with our previous observation of the lack of association of the presence of the *UGT1A9 –118T*$_{10}$ allele with UGT1A9 protein content in liver microsomes (Girard et al 2004).

When evaluating combined *UGT1A1/1A9* haplotypes, we did observed a trend toward lower formation of SN-38G in livers with the diplotype I/III (9/10 at -118) compared to those with I/I (10/10 at -118) whereas Innocenti *et al.* observed higher, but not significant, SN-38G formation in livers with the *UGT1A1/1A9* haplotype (diplotype I/III) compared tot those with diplotype I/I (Innocenti et al., 2005). In both studies, the sample size was rather limited to draw definitive conclusion. Nevertheless, overall *in vitro* data would point out to a limited involvement of this variant in the control of *UGT1A9* gene expression and raise the possibility of an effect on SN-38 glucuronidation through linkage with *UGT1A1* variants or to unknown polymorphism(s). This however, deserves to be verified since the experimental data do not currently provide the support to this hypothesis.

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A lack of LD between *UGT1A1 –53(TA)$_n$* and *UGT1A9 -118T$_n$* (r^2=0.26), and with the *UGT1A9 I399* (r^2=0.05) was observed in Caucasians. This is consistent with results of a recent study by Innocenti *et al.* (Innocenti et al., 2005). In this study, the LD between the *UGT1A1* -3279 and *UGT1A9 -118T$_n$* was however higher (r^2=0.39). When considering additional variants such as the *UGT1A9 -331/-440*, a strong LD (r^2=0.79) is observed between *UGT1A9* and *UGT1A1* (*-53(TA)$_n$*. This indicates that linkage among the common *UGT1* variants extend from *UGT1A1* through to at least *UGT1A9*, corresponding to a genetic distance of about 88 kilobases. A significant correlation between expression of both proteins in the liver was observed (R_s = 0.48; p=0.0006). We also observed that among the common *UGT1A1//1A9* haplotypes, polymorphisms associated with lower levels of UGT1A1 and UGT1A9 proteins appear on the same haplotype, indicating that subjects with low levels of UGT1A9 are also susceptible to have lower expression of UGT1A1.

Clearly, our genotyping results in limited human livers are exploratory in nature; therefore, our results should be interpreted with caution while awaiting replication and further investigation through clinical study. Nevertheless, this study indicate that the novel intronic *UGT1A9 I399* and the *-118T* promoter variation may represent additional candidates in combination with *UGT1A1* promoter haplotypes to predict SN-38 glucuronidation profile *in vivo*.

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FOOTNOTES

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LEGENDS TO FIGURE

Figure 1. Correlation analyses of *UGT1A9* intronic I399 variant genotypes with SN-38 glucuronidation activities (A), UGT1A9 protein expression (B), UGT1A1 protein expression (C) and the UGT1A9 specific substrate propofol (D).

Figure 2. Haplotype structure of *UGT1A9* determined with the PHASE program in Caucasian subjects only (n=42). Haplotypes with at least one genotype with less than 90% confidence were excluded. *Represents previously unreported polymorphisms.

Figure 3. Pairwise linkage disequilibrium (LD) R^2 for the *UGT1A1* and *UGT1A9*. The LD was calculated with the LDplotter program. The color tone indicates the extent of LD between the two loci.

Figure 4. Correlation analyses of *UGT1A9* -118T_n genotypes with SN-38 glucuronidation activities (A), UGT1A9 protein expression (B) and UGT1A1 protein expression (C). The horizontal bars indicate mean values. Relative luciferase activities for pGL3, *UGT1A9* T_9 and *UGT1A9* T_{10} constructs in HepG2 cell line are illustrated (D) (* p=0.004).

Figure 5. SN-38 glucuronidation activities in subjects with the *UGT1A1 -53(TA)$_{6/6}$* genotype stratified by UGT1A9 I399 C>T genotypes. The horizontal bars indicate mean values.

Figure 6. UGT1A9 protein expression (A) and SN-38 glucuronidation activities (B) associated with *UGT1A9* diplotypes, determined by PHASE program analysis in Caucasian subjects only. The horizontal bars indicate mean values. N corresponds to the number of subjects in this group. Only diplotypes having n≥3 measurements are reported. Significant results are shown.

Figure 7. Haplotype structure for *UGT1A1* (A) determined with the PHASE program in Caucasian subjects only (n=42). Haplotypes with at least one genotype with less than 90% confidence were excluded. UGT1A1 protein expression (B) and SN-38 glucuronidation activities (C) associated with *UGT1A1* diplotypes. The horizontal bars indicate mean values. N corresponds to the number of subjects in this group. Only diplotypes having n≥3 measurements are reported. Significant results are shown.

Figure 8. Haplotype structure of *UGT1A9-UGT1A1* (A) determined with the PHASE program in Caucasian subjects only (n=42). Haplotypes with at least one genotype with less than 90% confidence were excluded. *Represents previously unreported polymorphisms. SN-38 glucuronidation activities (B) associated with *UGT1A9-UGT1A1* diplotypes. The horizontal bars indicate mean values. N corresponds to the number of subjects in this group. Only diplotypes having n≥3 measurements are reported. Significant results are shown.

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Table 1: Primers used for amplification, sequencing and directed mutagenesis.

Primers	Sequences
R503	5'-ctaaattccagcaggctatc-3'
F506	5'-gtaggtcttttacatttccc-3'
R507	5'-cctgaaacagcaaaaccaa-3'
R670-HindIII	5'-ctagctgaagcttatcagagaactgcagctgaga-3'
F838	5'-ttgcttccaagtctttg-3'
R839	5'-attggggcactattcacaaaag-3'
F840	5'-tttacagtcccacaaaaacaag-3'
R841	5'-tcagatgagtagattccaaaatt-3'
F842	5'-acctcagttttagtttgattacc-3'
F1121-XhoI	5'-ctagcagctcgagttgggtaaatcattgtcagtgac-3'
F1154	5'-gagatcaaatttcaacacttg-3'
F1155	5'-tcagtgactgatttttttttttatgaaaggat-3'
F1159	5'-gcagcatgttttacaatcctttg-3'
R1164	5'-gtagattccaaaattttctc-3'
R1165	5'-catgtacagactggtaaag-3'
R1166	5'-atcctttcataaaaaaaaaatcagtcactga-3'
F1293	5'-caccttaagaatacttcacctttgg-3'
R1298	5'-caactaagataaaagcaagaatatgg-3'
F1473-SmaI	5'-ctagcagcccgggctcatatattcttgttcttttg-3'
R1474-SmaI	5'-ctagcagcccgggcagagaactgcagctgaga-3'

Restriction sites are underlined.

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Table 2 : UGT1A1 and UGT1A9 protein expression and glucuronosyltransferase activities in human livers genotyped for functional polymorphisms.

Functional polymorphisms	Number of subjects	UGT activities			UGT protein content (arbitrary units)	
		SN-38G pmoles/min/mg	Bilirubin-G pmoles/min/mg	E2-3G nmoles/min/mg	UGT1A1 protein expression	UGT1A9 protein expression
UGT1A1						
UGT1A1 -53						
*1/*1	24	63.2 ± 43.4	0.79 ± 0.46	0.38 ± 0.28	19.7 ± 8.4	
*1/*28	17	51.2 ± 45.9	0.58 ± 0.40	0.33 ± 0.29	15.0 ± 10.9	
*28/*28	7	36.6 ± 49.2 *	0.47 ± 0.68 *	0.22 ± 0.42*	8.3 ± 9.6*	
p value[a]		0.02	0.01	0.01	0.003	-
UGT1A1 -3156						
G/G	29	64.2 ± 44.9	0.79 ± 0.46	0.38 ± 0.29	19.9 ± 8.4	
G/A	12	43.6 ± 40.9	0.48 ± 0.33	0.31 ± 0.27	12.7 ± 10.9*	
A/A	7	36.6 ± 49.2 *	0.47 ± 0.68 *	0.22 ± 0.42*	8.3 ± 9.6*	
p value[a]		0.01	0.006	0.01	0.0006	-
UGT1A1 -3279						
T/T	13	65.5 ± 40.5	0.81 ± 0.34	0.43 ± 0.29	21.3 ± 6.7	
G/T	22	62.6 ± 49.3†	0.69 ± 0.52	0.34 ± 0.30	17.4 ± 11.3†	
G/G	13	31.9 ± 35.9*	0.49 ± 0.52*	0.25 ± 0.32*	9.9 ± 7.8*	
p value[a]		0.004	0.01	0.03	0.002	-
UGT1A9						
UGT1A9 -118						
$T_{9/9}$	18	39.2 ± 36.6	0.49 ± 0.45	0.25 ± 0.29	12.0 ± 8.7	7.7 ± 3.0
$T_{9/10}$	22	63.1 ± 48.0	0.72 ± 0.50	0.35 ± 0.29	18.3 ± 11.2	8.6 ± 3.2
$T_{10/10}$	8	68.7 ± 49.5	0.94 ± 0.40*	0.50 ± 0.34*	21.2 ± 6.3*	8.9 ± 3.0
P value[a]		0.05	0.02	0.03	0.03	0.5
UGT1A9 -275						
T/T	41	56.5 ± 46.0				7.8 ± 2.8
T/A	7	46.6 ± 41.7				11.2 ± 3.4
A/A	0	-				-
p value[b]		0.6	-	-	-	0.006
UGT1A9 -2152[c]						
C/C	43	55.5 ± 45.2				7.8 ± 2.7
C/T	5	51.0 ± 49.9				12.7 ± 2.5
T/T	0	-				-
p value[b]		0.7	-	-	-	0.0004
UGT1A9 I399[d]						
C/C	17	33.9 ± 25.2	0.48 ± 0.29	0.20 ± 0.20	13.5 ± 7.4	7.5 ± 3.0
C/T	20	54.1 ± 41.3	0.64 ± 0.48	0.37 ± 0.33*	15.4 ± 10.5	8.1 ± 2.5
T/T	11	89.5 ± 57.0*	1.01 ± 0.58*	0.50 ± 0.33*	22.7 ± 11.3*	10.1 ± 3.7*
P value[a]		0.007	0.05	0.006	0.12	0.08

Data are expressed as mean ± SD

[a] ANOVA test

[b] T-test

[c] Alleles with -2152T also included polymorphisms at positions -275T>A, -4549T>C and -5366G>T.

[d] Alleles with I399 also included polymorphisms at position I219 and I313.

* Significantly different (Student's t-test) from the corresponding homozygote reference group ($p<0.05$).

† Significantly different (Student's t-test) from the corresponding homozygote mutant group ($p<0.05$).

All other genotype-phenotype relationship were non-significant ($p>0.05$)

Figure 1

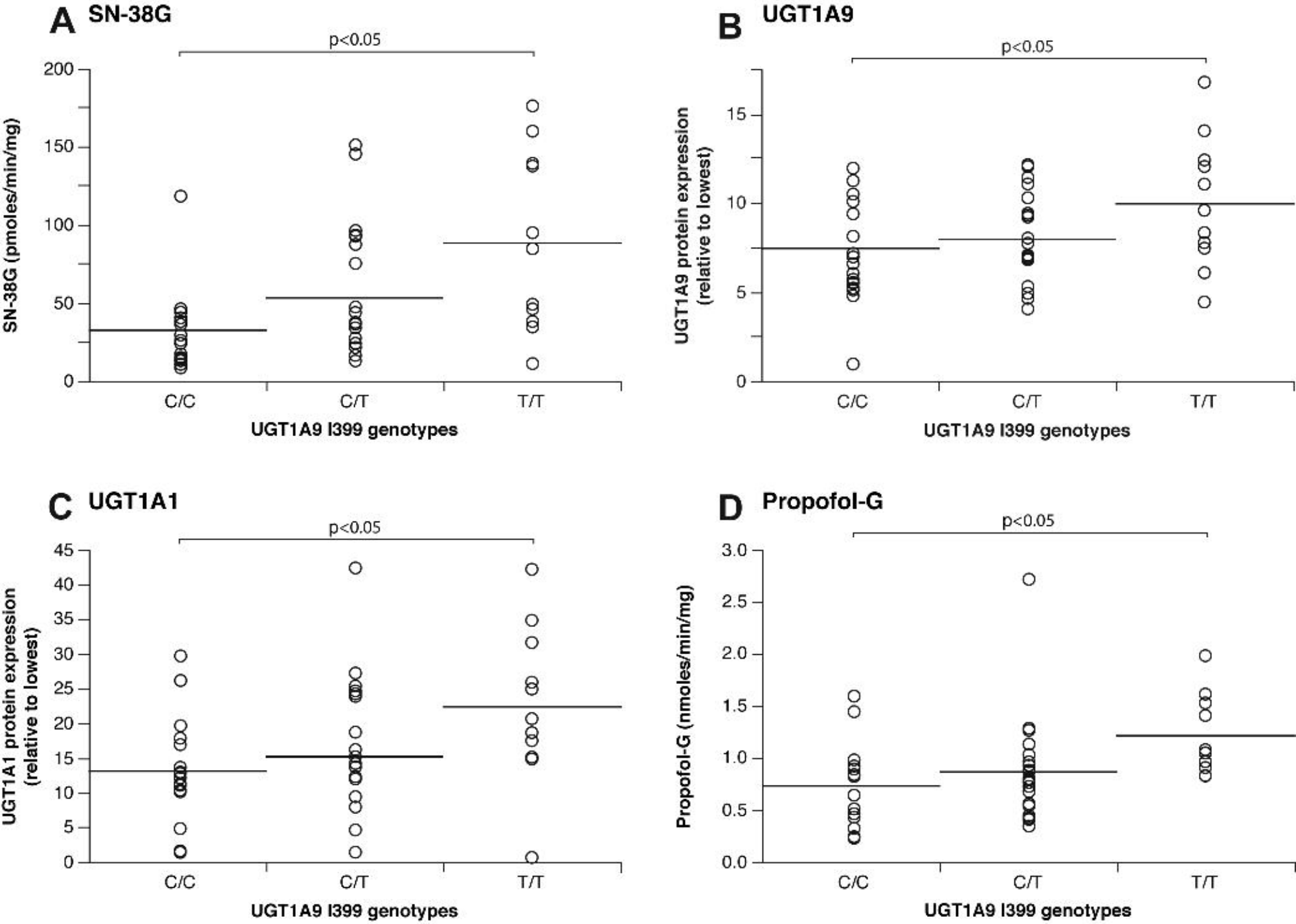

A
SN-38G
p<0.05
SN-38G (pmoles/min/mg)
200
150
100
50
0
C/C
C/T
T/T
UGT1A9 I399 genotypes
B
UGT1A9
p<0.05
UGT1A9 protein expression (relative to lowest)
15
10
5
0
C/C
C/T
T/T
UGT1A9 I399 genotypes
C
UGT1A1
p<0.05
UGT1A1 protein expression (relative to lowest)
45
40
35
30
25
20
15
10
5
0
C/C
C/T
T/T
UGT1A9 I399 genotypes
D
Propofol-G
p<0.05
Propofol-G (nmoles/min/mg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C/C
C/T
T/T
UGT1A9 I399 genotypes

Figure 2

UGT1A9 Haplotypes	UGT1A9 polymorphisms -5366* G › T	-4549* T › C	-2152 C › T	-275 T › A	-118 $(dT)_{9›10}$	I399* C › T	Frequency Caucasians
I	G	T	C	T	9	C	0.46
II	G	T	C	T	10	T	0.33
III	G	T	C	T	9	T	0.13
IV	G	T	C	T	10	C	0.05
V	T	C	T	A	9	T	0.03
VI	T	C	T	A	9	C	0.01

Figure 3

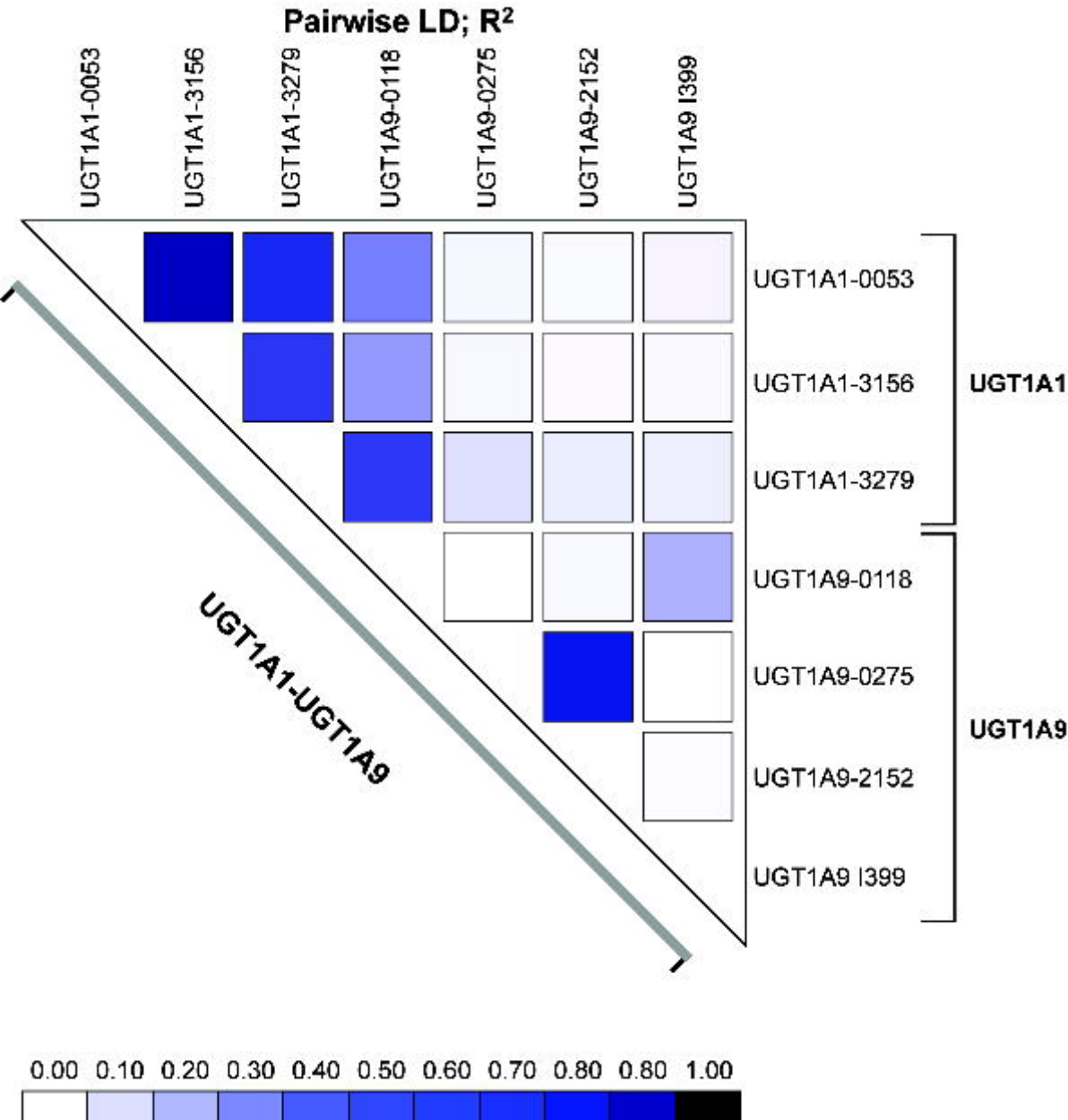

Pairwise LD; R²
UGT1A1-0053
UGT1A1-3156
UGT1A1-3279
UGT1A9-0118
UGT1A9-0275
UGT1A9-2152
UGT1A9 I399
UGT1A1
UGT1A9
UGT1A1-UGT1A9
0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.80 1.00

Figure 4

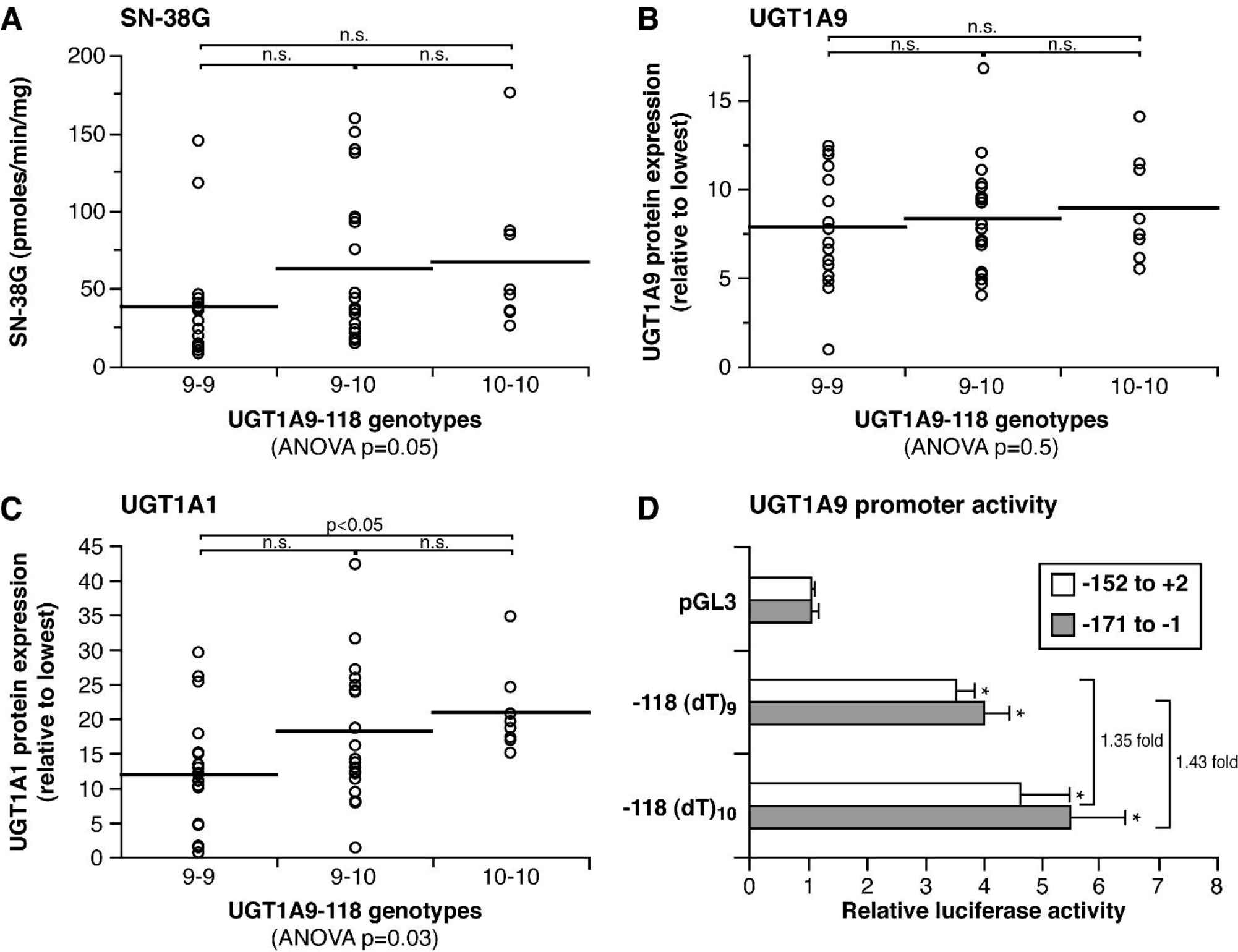

A
SN-38G
n.s.
n.s.
n.s.
200
150
100
50
0
SN-38G (pmoles/min/mg)
9-9
9-10
10-10
UGT1A9-118 genotypes
(ANOVA p=0.05)
B
UGT1A9
n.s.
n.s.
n.s.
15
10
5
0
UGT1A9 protein expression
(relative to lowest)
9-9
9-10
10-10
UGT1A9-118 genotypes
(ANOVA p=0.5)
C
UGT1A1
p<0.05
n.s.
n.s.
45
40
35
30
25
20
15
10
5
0
UGT1A1 protein expression
(relative to lowest)
9-9
9-10
10-10
UGT1A9-118 genotypes
(ANOVA p=0.03)
D
UGT1A9 promoter activity
-152 to +2
-171 to -1
pGL3
-118 (dT)9
-118 (dT)10
*
*
*
*
1.35 fold
1.43 fold
0
1
2
3
4
5
6
7
8
Relative luciferase activity

Figure 5

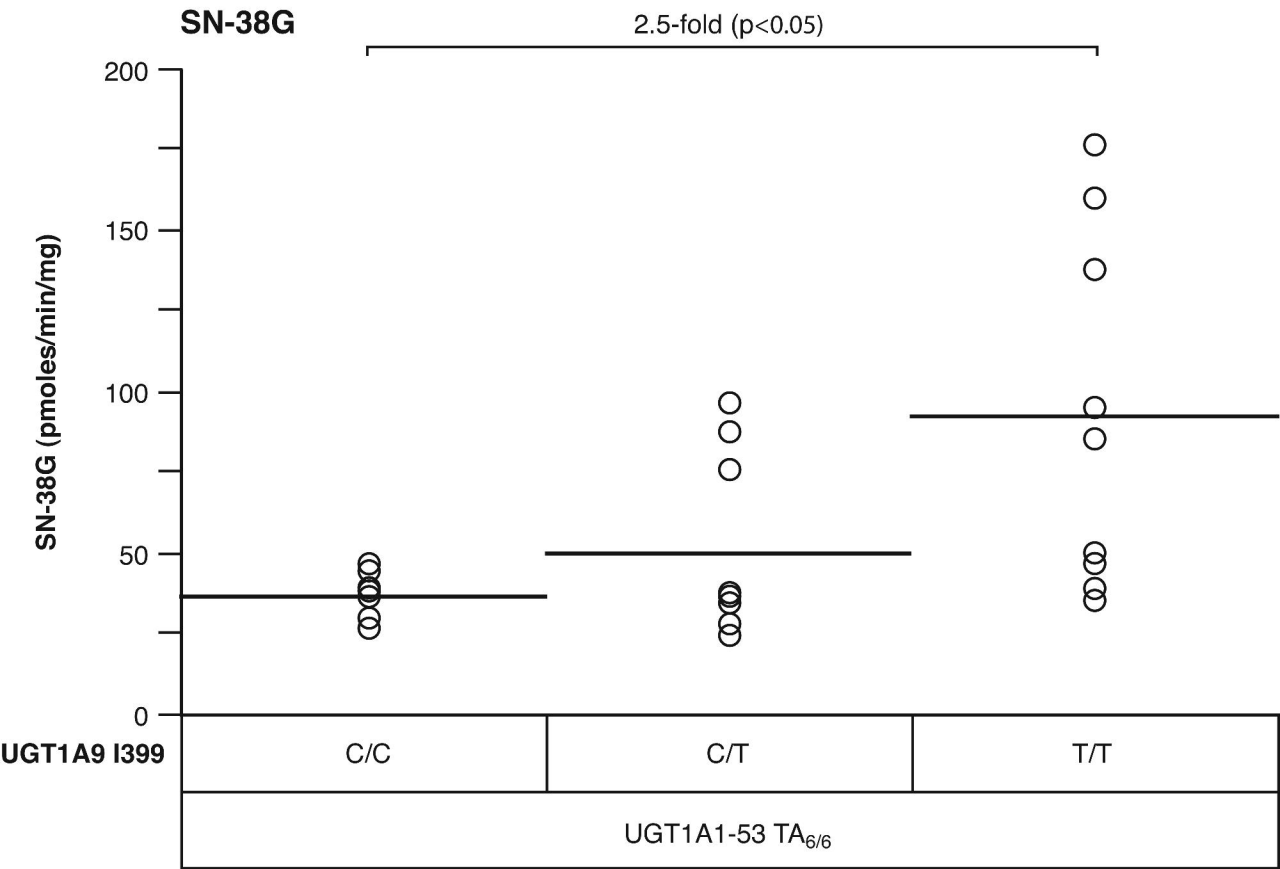

SN-38G
2.5-fold (p<0.05)
200
150
100
50
0
SN-38G (pmoles/min/mg)
UGT1A9 I399
C/C
C/T
T/T
UGT1A1-53 TA6/6

Figure 6

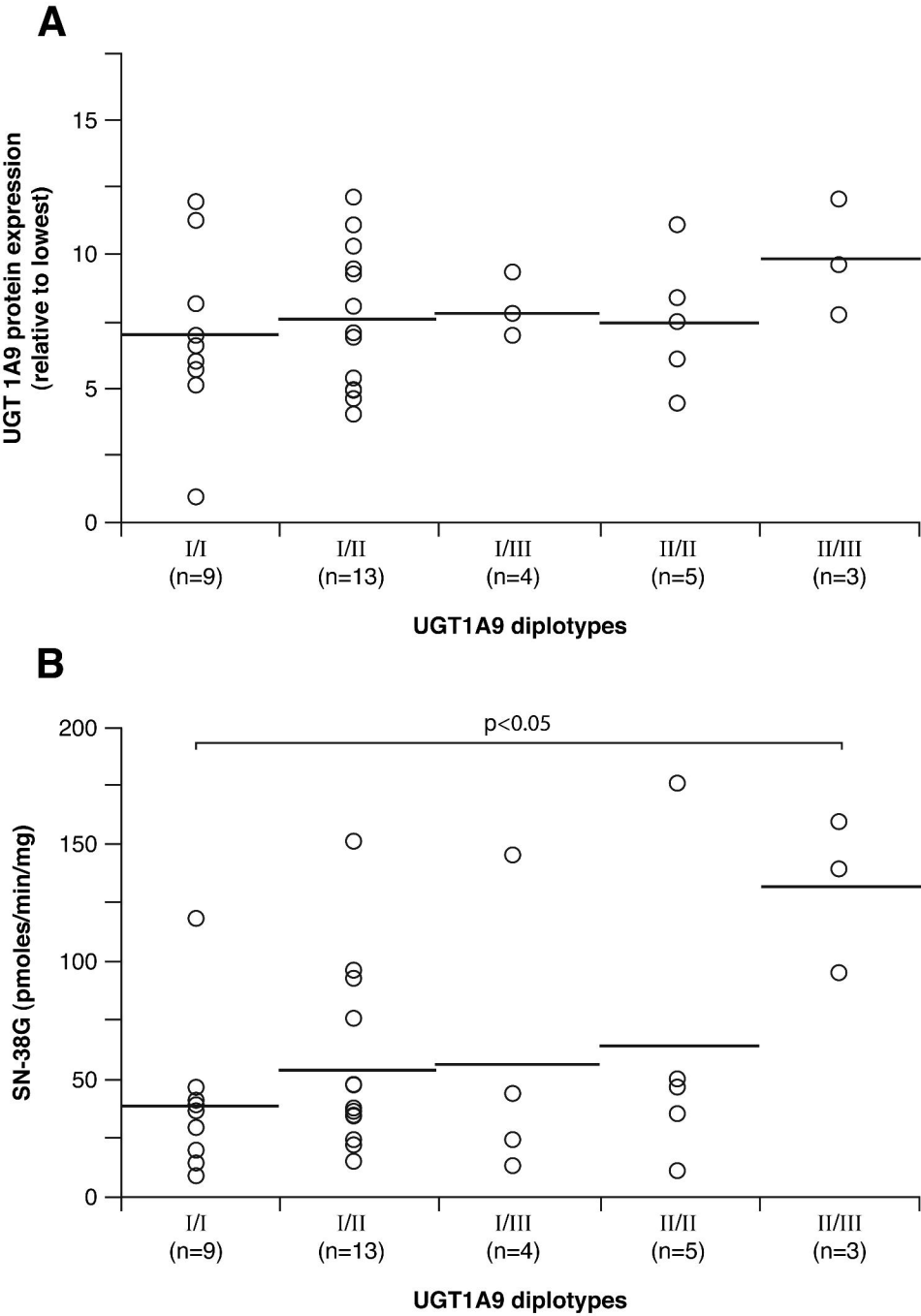
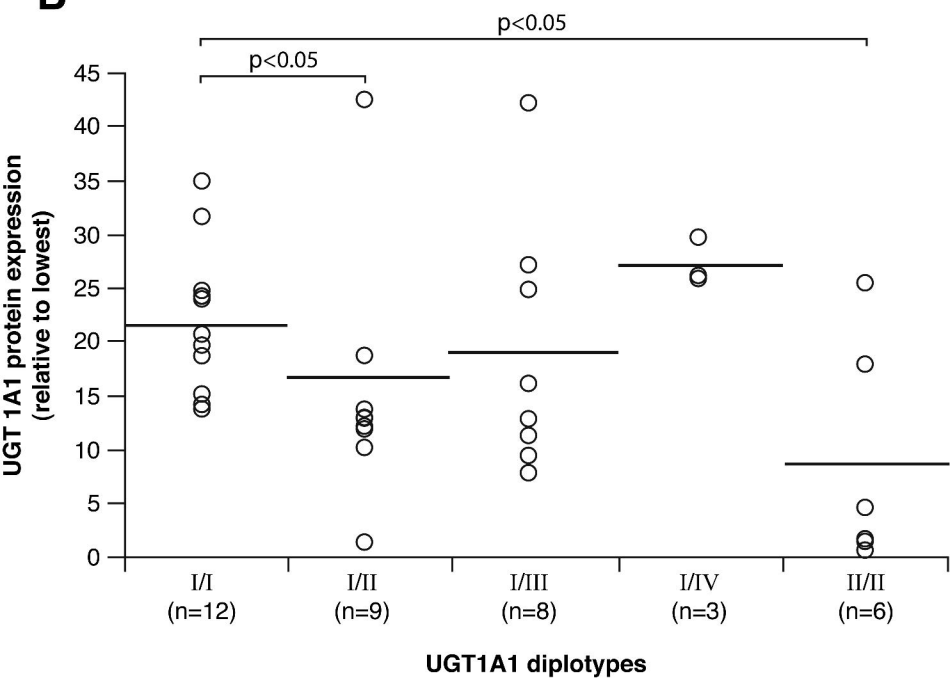

A
UGT 1A9 protein expression (relative to lowest)
15
10
5
0
I/I (n=9)
I/II (n=13)
I/III (n=4)
II/II (n=5)
II/III (n=3)
UGT1A9 diplotypes
B
p<0.05
SN-38G (pmoles/min/mg)
200
150
100
50
0
I/I (n=9)
I/II (n=13)
I/III (n=4)
II/II (n=5)
II/III (n=3)
UGT1A9 diplotypes

Figure 7

A

UGT1A1 Haplotypes	UGT1A1 polymorphisms: -3279 T › G	-3156 G › A	-53 $(TA)_{6›7}$	Frequency Caucasians
I	T	G	6	0.54
II	G	A	7	0.28
III	G	G	6	0.15
IV	G	G	7	0.04

B



C

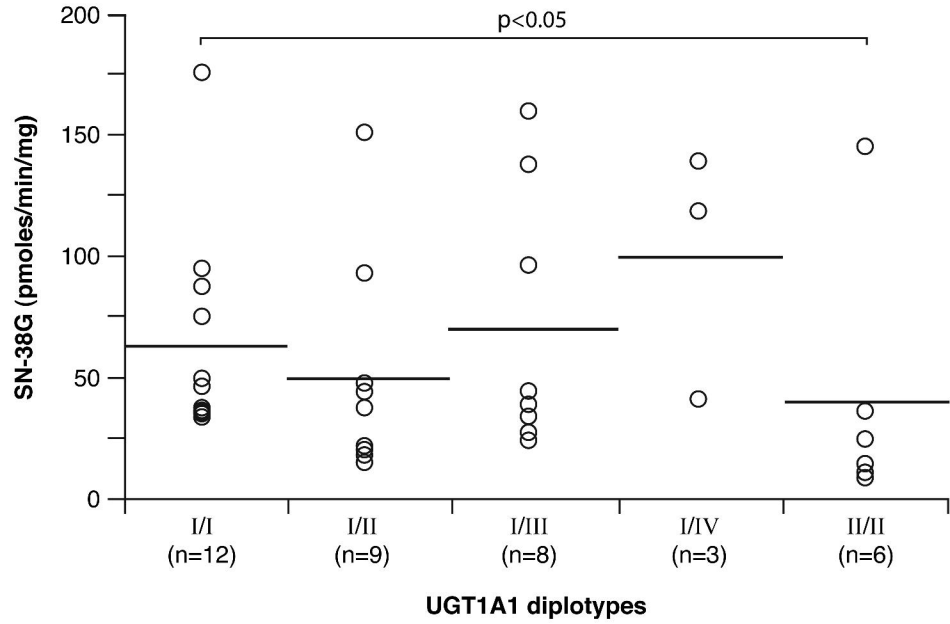


Figure 8

A

UGT1A9-UGT1A1 Haplotypes	UGT1A9 -5366* G › T	UGT1A9 -4549* T › C	UGT1A9 -2152 C › T	UGT1A9 -275 T › A	UGT1A9 -118 $(dT)_{9\text{›}10}$	UGT1A9 I399 C › T	UGT1A1 -3279 T › G	UGT1A1 -3156 G › A	UGT1A1 -53 $(TA)_{6\text{›}7}$	Frequency Caucasians
I	G	T	C	T	[10]	[T]	T	G	6	0.34
II	G	T	C	T	9	C	[G]	[A]	[7]	0.27
III	G	T	C	T	9	C	T	G	6	0.14
IV	G	T	C	T	9	[T]	[G]	[A]	[7]	0.06
V	G	T	C	T	[10]	C	T	G	6	0.06
VI	G	T	C	T	9	C	[G]	G	6	0.03
VII	G	T	C	T	9	[T]	[G]	G	6	0.03
VIII	[T]	[C]	[T]	[A]	9	[T]	[G]	G	6	0.03
IX	G	T	C	T	9	[T]	T	G	6	0.02
X	[T]	[C]	[T]	[A]	9	C	[G]	G	6	0.02

B